

DATE FILED: 05/06/2009
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B8/B8 CIP

IN THE CANADIAN PATENT OFFICE

Examiner : M. Gillen
Applicant : Biogen, Inc.
Application No.: 374,378
Filed : April 1, 1981
For : DNA SEQUENCES, RECOMBINANT DNA
MOLECULES AND PROCESSES FOR PRODUCING HUMAN
FIBROBLAST INTERFERON-LIKE POLYPEPTIDES

AFFIDAVIT OF WALTER C. FIERS

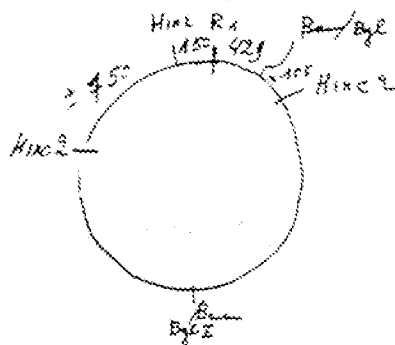
EXHIBITS 21-43

SUGANO EXHIBIT 1003
FIERS V. SUGANO
INTERFERENCE NO. 105,661

2

- 12 clones generated / mit 50%
zu schlechte Übergang

No 8 left sample for analysis



Opened Dec 30 2052
Decashelee le [Signature]
Commissioner of Patents
Commissaire des brevets
in presence of examiner [Signature]
en présence de l'examinateur [Signature]

2670

3750

next trip

Officials Harmon ~~PLC~~ - HFF 67-~~8~~ 8



This is EXHIBIT FIERs-21
to
the Affidavit of Walter C. Fiers
sworn before me
this 13th day of November, 2001

Proof.

Commissioner for Oath or Notary Public

of Zetach)

Label experiment net NF₁ pSRKB67-12 Δ19

pSTL24-8

NF₁ pBRKB-67

M5219 pSRKB67-12 Δ19

M5219 pSRKB-67

pSTL24-8

Vaccines: LB

Shift run Met medium (1/5 gefiltert)

light 1h to 22°C.

then shift run 42

Label periods

~~42°C~~ ~~Met medium~~ ~~run~~:

• SRKB67-12

↓

SRKB67-12 Δ19

} second NF₁ also M5219.

42°C : a) run 30-40 min
b) run 90-100 min
28°C : run 90-100 min

• STL24-8 on NF₁ on M5219

42°C : a) run 90-100 min
b) run 120-130 min
28°C : run 120-130 min

Label:

per tybomonte: 5 ml met 50 μ l ³⁵S Met

cellen opentrifugeren en supernatant in

100 μ l } 80 mM Tris pH 7,4

10% glycerol

totaal volume geschikt op \pm 150 μ l

Verspreiding

A) Rechtstreeks op gel

15 μ l cellen + 15 μ l 2 x Laemmli buffer
laaten op 47,5% verspreiden

gebruik van alle opstellingen 57L24-8

Δ19

SRKB (7-12)

B) Immunoprecipitatie met collidige cellen

15 μ l cellen + 15 μ l } 80 mM Tris
10% glycerol
2% SDS } 5 min koken

+ 400 μ l H₂O

+ 100 μ l NETS + BSA

Verzocht met Trellering
 Female pellet 3x met RETS gewassen
 Immunoprecipitaat geelend door biter met
 18.5% in aanwezigheid van Stage A.

→ Uitgeleid van 57429-8
 419.

C) Comotische Schoorten

45 µl cellen centrifugeren en droog in
 400 µl 20% Sucrose-EDTA

Klassieke Shock proceduren

Verwerking van cSS (na 400 µl).

- 130 µl geprecipiteerd met 10 µg Cytochrome c-antigeen
 en biter op 12,5% reageerend
- 60 µl cSS van immunoprecipitatie
 cf. Patrick

D) Openingstechnieken

Beoekeling is de fractie van een gemaakte
buisen na te gaan bij verschillende openingen
voorwaarden.

Werd uitgevoerd op vandenroeten 42° en D19
STL24-8

8 µl van gemaakte materiaal werd gemengd met
1 ml OV cultuur SRKB (niet gelabeld).
- gepelletiseerd in Eppendorf en ingevroren
bij -80°C

Methoden

1°) Opening met Lysosyme

Pellet in 100 µl HERES buffer / 1 mg/ml Lys
3 mM β -HE

- 30 min op 4°
- 1 x vieren CO₂- methanol
- ontlossen 37°C
- 30 min 20.000 rpm SS34 veldvries

Supernatans: - 20 µl afgenomen + 20 µl extract
→ 5cl

- rest naar immunisatie

2°) Opening met liposyme gevolgd door 0,1% SDS

- type zeals onder 1°)
- min clearing + 5 min tot 1, 1 1/2
(type licht beter te zien)
- clearing op zwk 30 min (groot niet zo goed; veel DNA vrij)
- venter zeals A.

3°) Opening door Sonificatie in centrale buffer

Pellet in 100 µl HEPES + 3 mM β-Me

Sonificatie op ijs met blinde tip in R.65 buisjes
10' met 15' antistijg; CX

Venter zeals 1°)

4°) Opening door Sonificatie in zure buffer

Pellet in 100 µl pH 2, buffer
of R.65 buisjes

Zeals 3°) en venter 1°)

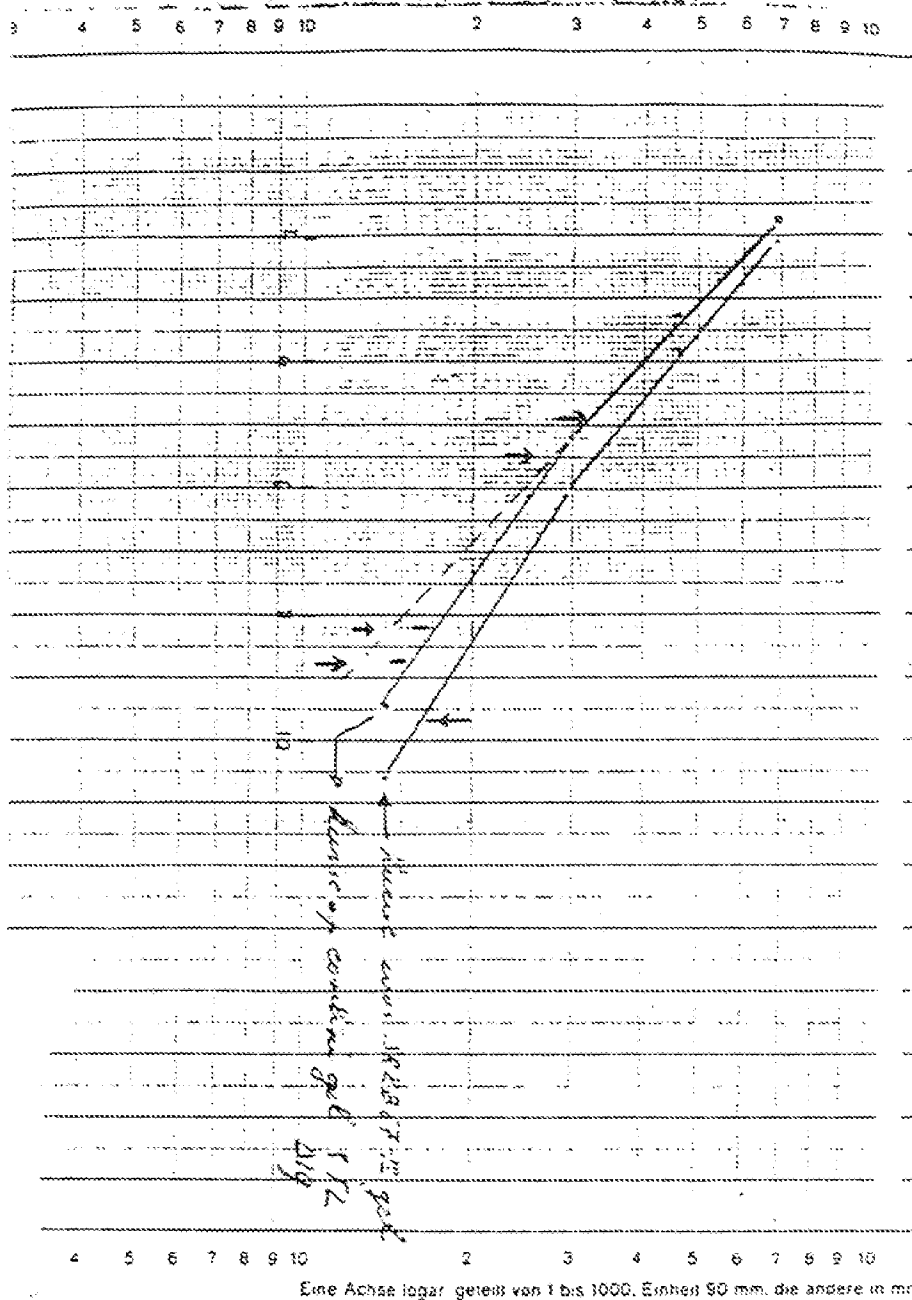
Linking gereintroduceerd met NaOH tot 0,02 M

Bepreking

Autoradiogramme van ontstekte cellen

Voor opmetingen die worden bij autoradiogramme
van kinetisch Experiment

Sequens van 115219 67-12



Eine Achse logar. geteilt von 1 bis 1000, Einheit 90 mm, die andere in mm

Vooropgelede lengte van precursorproducten

$\Delta 19$:
 23 aa β -lactamase leader
 59 aa mature β -lact tot aan HincII
 1 aa van HincII IF
 21 aa leader IF
 166 aa mature IF

Dec 30 2001
 M. G. Fiers

Totale Precursor: 270 aa 29110
 - β -lact-leader 247 aa 26676
 - Mature IF 166 aa 17928

27L24-8 : 33 aa van Replicase
 26 aa conif BglII tot aan ADE
 21 aa leader IF
 166 aa mature IF

Totale Precursor: 312 aa 33695

HS2
 TGG, BAT, CTT, CAE, TTT, CEE, AEE, CAA, CCT, TTC,
 GAA, GCC, TTT, GCT, CTE, ECA, CAA, CAE, ETA, ~~ETA~~
~~GGC~~, GAC, ACT, GTT, CET, GTT, GTC, AAC AUE
 GGC,

This is EXHIBIT FIERS-22

to

the Affidavit of Walter C. Fiers
 sworn before me
 this 19th day of November, 2001



Commissioner for Oath or Notary Public

Kinetisch experiment

11

M5219 p PLC HFIF 17-12 Δ19 (Δ19)
p PLC HFIF 17-8 (57L2)

Cellen in LB 28°C tot $2 \cdot 10^8$
Beweringen naar Met⁻ medium (~~filtrat~~).
Na 60 min shift... $t=0$

<u>Labeltyden</u> :	28°	30'	} 1 ml met 5 µl ²⁵ S gebruikte 10 min.
	42°	30'	
		60'	
		125'	
		150'	
		180'	
	22°	180'	

Cellen gekolktend door centrifugatie en
opgekoelt in 40 µl laemmli buffer

Gel: 10 µl geladen

Opened Dec 30 2002
 Décachetée le _____

[Signature]
 Commissioner of Patents
 Commissaire des brevets

In presence of examiner [Signature] J. H.
 en présence de l'examineur

This is EXHIBIT FIERS-23
 to
 the Affidavit of Walter C. Fiers
 sworn before me
 this 19th day of November, 2001



 Commissioner for Oath or Notary Public

Induktie

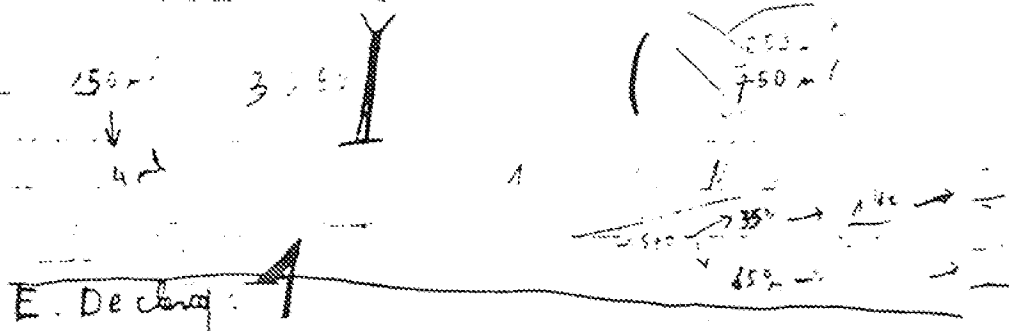
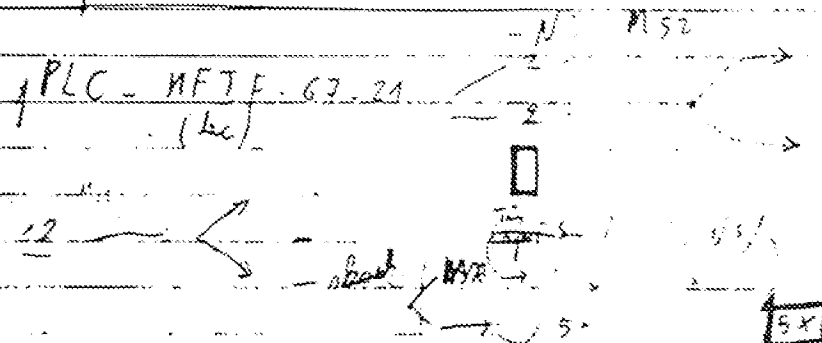
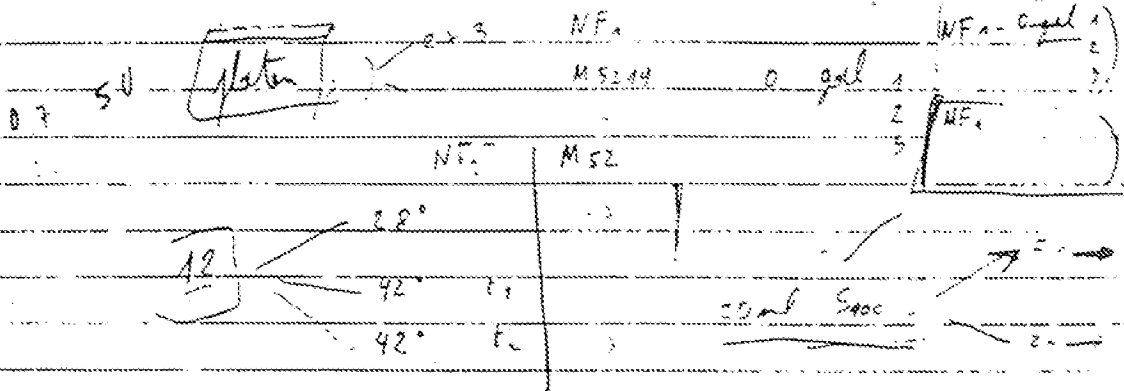
26/4/80

Na bestuderen van de resultaten van de tests met
leuren werden volgende inducties gepland:

1/ p. PLA - HFIF - 67 - 12 zowel in M5219
als in NF₁

2/ p. PLC - HFIF - 67 - 11 met las-operator.
in het min 2 [Ld]

ERIC S



Verslag van Proleptische resultaten

Er leit sein neues Kommando diesem die sein Leuten
in Brüssel. Nachher sei er bei einem „marchand“
rehabilitiert als voriger weihen.

Het voorname is dat en, handt niet in overeenkomst
met Brussel, ~~kan~~ onopgelijde topologische afwijking
pPla-HF. 67.12.
is in extracten van pSAB-67-12. 1ste
handelaar voor ~~re-injectie~~ Een kleine (0.2) afwijking
is aanwezig in de HF. 1 (na injectie), na meer
5 V/m (0.2) in de HF. 19 (na injectie). De 20°C korrel
perov $\epsilon = 2$. Het voorname is bij dat de
afwijking in E. 57 $\epsilon = 0$ is. Dit is wel het
voorname. De herhaling van het exp. met 2 glycerol
volgt volgende week.

7.5 Normaal deense volpende week penning
in ^(week) hennery. Ook was schitterend in het buitenland
^(tot vrijdagavond)
op vrijdag. Toen het koningspaar zijn dat de hennery
thuis konakkeren om ook de penning doorgeven
de hennery laten rijden, Biedenht.

Rk

(Zinbaar vandaan de pannen dak met
garanties)

Konstruktie met lac-Operator.

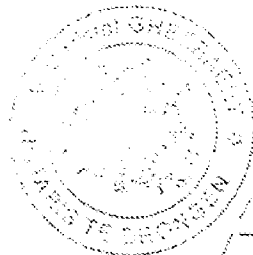
- ① Analyse van drie positieve kolonies
Slechts 1 bruikbare kandidaat. Hiervan wordt de
orientatie maandag bepaald.
Induktie gepland voor maandag.
- ② Hernemen konstruktie met lac-operator, na zuivering
van EcoRI fragment.
Konstruktie gemaakt in pPLa HFIF 67-1 wegens gebrek
aan DNA van pPLC HFIF-67-11.
Transformatie gebeurt maandag in K12.

E. Seeman.

Dec 30 02

W. C. Fiers

in presence of *M. J. Hill* *J. H. B.*
 en présence de l'examinateur



This is EXHIBIT FIERS-24
 to
 the Affidavit of Walter C. Fiers
 sworn before me
 this 13th day of November, 2001

Commissioner for Oath or Notary Public

- preparation of wafers
 - cleaning - Hyper-H 7.0
 - 1.5% self-forming
 - 3 min @ 60°C
 - AmSO₄ - number of plates - actual vol. of product
 (vol. of 1.5% in 1 of 1000 ml)
 - structure of high-molecular solution - ST 6.7-12 - 100% cellulosic
 ST 6.7-12 - 100% cellulosic
 ST 6.7-12 - 100% cellulosic

	of ST 6.7	of ST 6.7
1/ ST 6.7-11 / NF 1 / 1.5% C	2.10	2.2
4/ ST 6.7-11 / NF 1 / 1.5% C	2.10	2.2
5/ ST 6.7-12 / NF 1 / 1.5% C	2.10	2.2
6/ ST 6.7-11 / DS 24 / 2.8% C	2.10	2.2
7/ ST 6.7-11 / DS 24 / 4.1% C	2.10	2.2
8/ ST 6.7-12 / DS 24 / 4.1% C	2.10	2.2
9/ ST 6.7-12 / NF 1 / 1.5% C	2.10	2.2
10/ ST 6.7-12 / NF 1 / 1.5% C	2.10	2.2
11/ ST 6.7-12 / DS 24 / 4.1% C	2.10	2.2
12/ ST 6.7-12 / DS 24 / 4.1% C	2.10	2.2
13/ ST 6.7-12 / DS 24 / 4.1% C	2.10	2.2
14/ ST 6.7-12 / DS 24 / 4.1% C	2.10	2.2
15/ ST 6.7-12 / DS 24 / 4.1% C	2.10	2.2
16/ ST 6.7-12 / DS 24 / 4.1% C	2.10	2.2
17/ ST 6.7-12 / DS 24 / 4.1% C	2.10	2.2
18/ ST 6.7-12 / DS 24 / 4.1% C	2.10	2.2
19/ ST 6.7-12 / DS 24 / 4.1% C	2.10	2.2
20/ ST 6.7-12 / DS 24 / 4.1% C	2.10	2.2

17/ ST 6.7-12 / DS 24 / 4.1% C
 18/ ST 6.7-12 / DS 24 / 4.1% C
 19/ ST 6.7-12 / DS 24 / 4.1% C
 20/ ST 6.7-12 / DS 24 / 4.1% C

21/ ST 6.7-12 / DS 24 / 4.1% C
 22/ ST 6.7-12 / DS 24 / 4.1% C
 23/ ST 6.7-12 / DS 24 / 4.1% C
 24/ ST 6.7-12 / DS 24 / 4.1% C
 25/ ST 6.7-12 / DS 24 / 4.1% C
 26/ ST 6.7-12 / DS 24 / 4.1% C
 27/ ST 6.7-12 / DS 24 / 4.1% C
 28/ ST 6.7-12 / DS 24 / 4.1% C
 29/ ST 6.7-12 / DS 24 / 4.1% C
 30/ ST 6.7-12 / DS 24 / 4.1% C

Deception

• $\log_{10} y = \log_{10} x$ (and coefficient over 3... power diminished)
 for antilog of E_{10}

from the road of E. 187

in der Nähe der ... 25.11.19 (Brenn. etc.)
gla. (Linsen)

Geo. (Learney)

• morphologisches Institut am 8. Juni 12

SRU B-SP-16 - 100 - 100 - 100

12. 0. 2

2000

" under I^c & Ch 6 & our Hand Dy

... post^o negat^o in a 54/.

Am 04/11/1914

all expenditure as well
as income are treated

besluit = over het E.S.O.T. u h.t. u hebt een conclusie
= macroeconomische conclusies op T₂, maar problemen
op T₁ en T₂ met
man kan denken we het interpreteren?

~~ap. 57~~ ~~human~~ ~~von op. below~~ ~~best 2L~~ ~~schaden~~ ~~von~~
~~in E. 57~~ ~~kanalende~~ ~~abstrakt~~ ~~von~~ ~~in~~ ~~kanalende~~
~~abstrakt~~ ~~von~~ ~~p. 57~~

1/ ~~best 57~~ ~~7mm~~ ~~2.2mm~~ ~~785~~ ~~4°C~~
 2/ ~~kanalende~~ ~~abstrakt~~ ~~2L~~ ~~von~~ ~~mit~~ ~~5mm~~ ~~p. 57~~
 3/ ~~funktion~~ ~~von~~ ~~2.2mm~~ ~~p. 57~~

~~ap. 57~~
~~funktion~~
 1/ 2.5
 2/ 2.2
 3/ 2.05
 4/ 2.05
 5/ 2.05

best 57 in kanalende ~~von~~
 1/ ~~kanalende~~ ~~abstrakt~~ ~~in~~ ~~mit~~ ~~2L~~ ~~von~~ ~~best 57~~
 2/ ~~kanalende~~ ~~abstrakt~~ ~~2L~~ ~~von~~ ~~mit~~ ~~5mm~~ ~~p. 57~~
 3/ ~~funktion~~ ~~von~~ ~~2.2mm~~ ~~p. 57~~

1/ 1.7
 2/ 1.2
 3/ 0.7
 4/ 0.5
 5/ 0.5
 6/ 0.5
 7/ 0.5
 8/ 0.5
 9/ 0.5
 10/ 0.5
 11/ 0.5
 12/ 0.5

best 57 in kanalende
 1/ ~~kanalende~~ ~~abstrakt~~ ~~in~~ ~~mit~~ ~~2L~~ ~~von~~ ~~best 57~~
 2/ ~~kanalende~~ ~~abstrakt~~ ~~2L~~ ~~von~~ ~~mit~~ ~~5mm~~ ~~p. 57~~
 3/ ~~funktion~~ ~~von~~ ~~2.2mm~~ ~~p. 57~~

3/ ~~AcH 54~~ ~~6.7mm~~ ~~h=2.0mm~~ ~~0.0517~~ ~~polymer-HCL~~ ~~pH 2.2~~
 5.00 ~~apH 3~~ ~~2.5~~ ~~phthalate~~
 fraction = 15 drops per 2 reported

ind. #	ESM	TL	AD	IDS
1/	<1.0	CO2	3.0	in / middle
2/	<1.0	CO2	3.0	up / end
3/	2.5	2.7	3.0	
4/	2.0	2.7	3.0	
5/	2.0	2.0	2.5	
6/			2.5	
7/			2.5	
8/			2.5	
9/			2.5	
10/			2.5	
11/			2.5	
12/			2.5	

4 Sephadex 450 6.7mm h=2.0mm 0.0517 2 SDS, h.c
 Size = pH 7 + 2P gelatin
 fraction = 15 drops per 2 reported
 no. SDS very thick in pres. with Ac up

ind. no	apH, 5M	ap TL	CO2	AD
1/	<1.0	CO2	3.0	
2/	<1.0	CO2	3.0	
3/	<1.0	CO2	3.0	
4/	<1.0	CO2	3.0	
5/	<1.0	CO2	3.5	
6/			3.5	

5/ AcH 54 6.7mm h=2.0mm 0.0517 polymer-HCL pH 2.2
 Size = pH 7 + 2P, h=5.0mm pH 2.2, ~~polymer~~
 fraction = 15 drops per 2 reported

ind. no	apH, 5M	ap TL	CO2	AD
1/	<1.0	CO2	3.0	
2/	<1.0	CO2	3.0	
3/	<1.0	CO2	3.0	
4/	1.0	CO2	3.0	
5/	1.7	1.7	2.5	
6/	2.0	1.5	3.0	
7/			2.5	
8/			2.0	
9/			2.0	
10/			2.0	
11/			2.0	
12/			2.0	

6/	Sept. 65				
		all up 5			
		op E. 57	op 21		
2/	15		1.2	dr. line	3.5
6/	15		1.5		2.0
3/	22		0.9		1.0
4/	26.0		20.2		20.2
1/	2.12		2.12		2.12
6/					2.0

Sept 20. High water 1

• $\log_2 1 = 2^0 = 1$ and $\log_2 2 = 2^1 = 2$

(B) Log. viz. much ground pd. than SDS run across the lake
any more further in time or better; do you enjoy them

[illegible]

~ E.g.: "My name is ... I am ... I live ..."

[illegible]

⑥ unbenannt 1000 m 19. 10. 1971 } 5. 10. 1971 / Exponat-Hilf. pH 6.2
+ 12. 10. 1971

Strömungslinie des 2. n

5. 2

Jan 24/90. 78.

~~the~~ ~~longer~~ ~~the~~ ~~more~~ ~~it~~ ~~is~~ ~~likely~~ ~~that~~ ~~the~~ ~~more~~ ~~it~~ ~~will~~ ~~be~~ ~~seen~~ ~~to~~ ~~be~~ ~~a~~ ~~very~~ ~~good~~ ~~one~~ ~~at~~ ~~all~~ ~~times~~ ~~(C)~~

Am 504 / - 0.7 mls. lysaat
+ 2.0 ml 5% Am 504 verdunding
20' 40

— *expanding from long fine. Hammering.*

gutter 785

see also, same in 731 of 1131 (book)

[illegible]

other by me 425-0016525 (K.H.)

penelope the 4th

200 = 500 gal - 300 gal 4.44

and expanding on the things
in more than one

patents for drinks of Lollapalooza

- ① Lac 2 - 28°C
 - ② Lac 2 - 42°C
 - ③ Lac 4 - 28°C
 - ④ Lac 4 - 42°C
 - ⑤ PSR 067-12 28°C
 - ⑥ PSR 067-12 42°C
 - ⑦ SRH 08 - 42°C
- 250 gals. of 1A ~~water~~ 10% Lollapalooza

Results vs 2000 2001 2002 2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016 2017 2018 2019 2020 2021 2022 2023 2024 2025 2026 2027 2028 2029 2030 2031 2032 2033 2034 2035 2036 2037 2038 2039 2040 2041 2042 2043 2044 2045 2046 2047 2048 2049 2050 2051 2052 2053 2054 2055 2056 2057 2058 2059 2060 2061 2062 2063 2064 2065 2066 2067 2068 2069 2070 2071 2072 2073 2074 2075 2076 2077 2078 2079 2080 2081 2082 2083 2084 2085 2086 2087 2088 2089 2090 2091 2092 2093 2094 2095 2096 2097 2098 2099 2100 2101 2102 2103 2104 2105 2106 2107 2108 2109 2110 2111 2112 2113 2114 2115 2116 2117 2118 2119 2120 2121 2122 2123 2124 2125 2126 2127 2128 2129 2130 2131 2132 2133 2134 2135 2136 2137 2138 2139 2140 2141 2142 2143 2144 2145 2146 2147 2148 2149 2150 2151 2152 2153 2154 2155 2156 2157 2158 2159 2160 2161 2162 2163 2164 2165 2166 2167 2168 2169 2170 2171 2172 2173 2174 2175 2176 2177 2178 2179 2180 2181 2182 2183 2184 2185 2186 2187 2188 2189 2190 2191 2192 2193 2194 2195 2196 2197 2198 2199 2200 2201 2202 2203 2204 2205 2206 2207 2208 2209 2210 2211 2212 2213 2214 2215 2216 2217 2218 2219 2220 2221 2222 2223 2224 2225 2226 2227 2228 2229 2230 2231 2232 2233 2234 2235 2236 2237 2238 2239 2240 2241 2242 2243 2244 2245 2246 2247 2248 2249 2250 2251 2252 2253 2254 2255 2256 2257 2258 2259 2260 2261 2262 2263 2264 2265 2266 2267 2268 2269 2270 2271 2272 2273 2274 2275 2276 2277 2278 2279 2280 2281 2282 2283 2284 2285 2286 2287 2288 2289 2290 2291 2292 2293 2294 2295 2296 2297 2298 2299 2300 2301 2302 2303 2304 2305 2306 2307 2308 2309 2310 2311 2312 2313 2314 2315 2316 2317 2318 2319 2320 2321 2322 2323 2324 2325 2326 2327 2328 2329 2330 2331 2332 2333 2334 2335 2336 2337 2338 2339 2340 2341 2342 2343 2344 2345 2346 2347 2348 2349 2350 2351 2352 2353 2354 2355 2356 2357 2358 2359 2360 2361 2362 2363 2364 2365 2366 2367 2368 2369 2370 2371 2372 2373 2374 2375 2376 2377 2378 2379 2380 2381 2382 2383 2384 2385 2386 2387 2388 2389 2390 2391 2392 2393 2394 2395 2396 2397 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several families live in the tent - in summer camp -
 several with our well-bred people. Nov 60 4/

7-2-2-3

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Ex. 7.1.22

[Faint handwritten notes at the bottom of the page]

E. coli, *L. monocytogenes*

[Faint handwritten marks]

$E_{\text{eff}} = 1/2 T_{\text{eff}}$

2-2

James L. Smith 1851

Amesbury, N. H., 4/25/51

4/100 ~~indicates~~ ~~from~~ ~~the~~ ~~same~~ ~~source~~ ~~as~~ ~~the~~ ~~STP~~ ~~, 219~~
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Methoden

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E. sp. simileschickellianus number: Hesperia 47: 55-56 m.
 Hesperia 47: 55-56 m.
 32. California

- shell specimen of - 8.5" - no individual data recorded - volume 1/2
 - shell from 104 / 50% of - 100% of - volume 1/2

- shell analysis - 735.0 m. - volume 1/3
 analysis - 1/1000 - Hesperia 47: 55-56 m.
 volume 1/4

- shell of 17. SDS - 12. 100% - 50% - volume 1/5
 1' - volume 1/6

analysis - 735 m. - volume 1/5

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2011/11/11

7-11-1944 4-14-44

10/15/2011

Federal Camp C 20	Female C 2	BEL C 2	PRK C 2	Hammond C 1	T 21 3.5	E. 307 V 2 15 825
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4/12/1978	C1.0	C2	C1	C2	C2	L2	L1	L0
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✓ *Asplenium platyneuron*

6/8 1944

Prof. J. B. Griffiths, Jr.

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re: 2.342

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 Commissaire des douanes
 In presence of *[Signature]*
 en présence de l'examinateur



This is EXHIBIT FIERS-25
 to
 the Affidavit of Walter C. Fiers
 sworn before me
 this 19th day of November, 2001

Commissioner for Oath or Notary Public

Expression of the human fibroblast interferon gene in *Escherichia coli*^a

(hybrid ribosome-binding site/antiviral activity/lacZ gene fusions/portable promoter)

TADATSUGU TANIGUCHI¹ AND
LEONARD GUARENTE¹, THOMAS M. ROBERTS², DAVID KIMELMAN², JOHN DOLHAN III², AND
MARK PTASHNE¹

¹Cancer Institute, Japanese Foundation for Cancer Research, Toshima-Ku, Tokyo 170, Japan; and ²The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

Contributed by Mark Ptashne, June 25, 1980

ABSTRACT We applied the method of Guarente *et al.* [Guarente, L., Lauer, C., Roberts, T. M., & Ptashne, M. (1980) *Cell* 20, 543-553] to construct plasmids that direct expression in *Escherichia coli* of the human fibroblast interferon (F-IF) gene. Two plasmids were recovered. One directs efficient synthesis of a protein whose primary sequence is that of pre-F-IF and the other, that of mature F-IF. Extracts of bacteria synthesizing mature F-IF display antiviral activity characteristic of human F-IF. This activity is lower than that expected from the differential rate of synthesis of the protein. We have detected no such activity in extracts of bacteria synthesizing pre-F-IF.

Human fibroblast interferon (F-IF) is a glycoprotein produced by human fibroblasts in response to virus and certain polynucleotides (1, 3). The secreted protein has potent antiviral activity that is readily assayed *in vitro* (2, 3). The sequence of the amino-terminal 15 amino acids of F-IF has been reported (4).

A cDNA molecule encoding human F-IF was cloned by Taniguchi *et al.* (5). The DNA sequence of this molecule predicts that the secreted F-IF contains 166 amino acids; the first 13 of which would be identical to the corresponding sequence of the protein as determined by Knight *et al.* (4). Moreover, the sequence is consistent with the idea that F-IF is synthesized as a precursor (pre-F-IF) with a 21-amino-acid hydrophobic leader at its amino terminus (5, 7).

A series of papers from this laboratory^a has developed methods to express cloned prokaryotic and eukaryotic genes in *Escherichia coli* (8-11). The protein products of these plasmid-carried genes were produced in their native states—that is, unfused to other proteins (8-11). The essential feature of this method is to position a "portable promoter" in front of the cloned gene so that the gene is efficiently transcribed and the resultant mRNA is efficiently translated, beginning at an in-phase start codon—e.g., AUG. This AUG may, but need not be, that which directs initiation of synthesis of the native protein *in vivo*. The procedure of Roberts *et al.* (9, 10) enables us to position the portable promoter at various positions in front of the cloned gene by using recombination *in vitro*. The method of Guarente *et al.* (11) exploits *lac* genetics to identify those positionings that direct efficient transcription and translation of the cloned gene. This latter procedure eliminates the need for any assay for the gene product to identify those bacteria that express the desired proteins (see *Method of Gene Expression in Results*).

The protein and DNA sequence data referred to above indicate that both F-IF and pre-F-IF bear methionine residues

at their amino termini (4, 6). We describe in this paper the application of the method of Guarente *et al.* (11) to the F-IF gene. We describe the construction and identification of plasmids that direct the efficient synthesis of two proteins. The primary sequences apparently correspond to the sequence of F-IF in one case and to that of pre-F-IF in the other. F-IF produced in bacteria prevents viral growth as assayed *in vitro*.

MATERIALS AND METHODS

DNA Constructions. All techniques were as described by Guarente *et al.* (11). pTR56 (see Fig. 1) was constructed in two steps as follows. First, a plasmid (pLG111) was constructed that bears a *Hind*III synthetic linker three nucleotides before the ATG of pre-F-IF (6). This was accomplished by joining four DNA fragments: (i) a *Bam*HI-*Pst*I backbone fragment from pLG300 (11); (ii) a *Hind*III-*Bgl*II fragment containing the entire F-IF coding sequence from TplF319-10 (6); (iii) a *Pst*I-*Pvu*II fragment from pGL101 bearing the 3' end of the *amp* gene; and (iv) a *Hind*III linker. Ligation of these fragments fuses two complementary sticky ends (*Pst*I-*Pst*I and *Bam*HI-*Bgl*II) and two blunt ends (*Pvu*II-*Hind*III linker and *Hind*III linker-*Hind*III). The *Pst*I joining thus reconstitutes *amp*. Second, pTR56 was constructed by joining three fragments: (i) a *Pst*I-*Pst*I fragment from pLG111 bearing the 3' portion of *amp* and the 5' portion of the F-IF gene; (ii) an internal fragment of the F-IF gene extending from the *Pst*I site to the first downstream *Hind*III site (6), the latter having been rendered flush by DNA polymerase I (12); and (iii) a *Pst*I-*Bam*HI fragment from pLG300 (11) that bears the 3' end of *amp* and a 3' fragment of *lacZ*. The *Bam*HI end of this fragment had been rendered flush by DNA polymerase I (12). Ligation of these fragments generates two *Pst*I-*Pst*I fusions, one of which reconstitutes *amp*, as well as a *Bam*HI (filled in)-*Hind*III (filled in) fusion joining the 5' portion of the F-IF gene in phase with the 3' portion of *lacZ* (cf. refs. 6 and 11).

Radiolabeling of Proteins. The procedure has been described (11). Pulse labeling was with 300 μ Ci (1 Ci = 3.7×10^{10} becquerels) of [³⁵S]methionine and chasing was achieved by adding a 1000-fold excess of unlabeled methionine. This was done at 30°C. Labeled extracts were run on 15% acrylamide gels for analysis as described (13).

Preparation of Bacterial Extracts. Extracts were prepared essentially as described by Nagata *et al.* (14) except phenylmethylsulfonyl fluoride and EDTA were added in the lysis

^aThe publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: F-IF, fibroblast interferon; SD, Shine-Dalgarno.
^aThe work described herein was carried out by the authors at The Biological Laboratories, Harvard University.



FIG. 2. (A) Nucleotide sequences of the DNAs around the regions encoding ribosome-binding sites of genes encoding pre-FIF (in pGL104R) and mature FIF (in pGL117R). The vertical lines separate sequences carried on the lac portable fragment from FIF sequences. The boxes indicate the lacZ SD sequence and the ATGs encoding the amino-terminal methionines of pre-FIF and of mature FIF. (B) Corresponding region of wild-type lacZ 117.

(Fig. 3C; see also Fig. 3I). The DNA containing the *lacZ* 3' gene fragment was removed and replaced with the 3' end of the F-IF gene, regenerating intact F-IF (pLC117R) and pre-F-IF (pLC104R), in which R indicates the reconstituted F-IF gene (Fig. 2D).

Plasmids that Direct the Synthesis of F-IF (pLC117R) and pre-F-IF (pLC104R). Fig. 3A shows the DNA sequence around the junctions of the portable promoter and the ATGs encoding the amino terminus of pre-F-IF (pLC104 and pLC104R) and that of mature F-IF (pLC117 and pLC117R). In each case, the SD sequence (ACGA) of *lacZ* carried on the portable promoter has been positioned seven base pairs from the ATG of F-IF. This is precisely the distance between the SD sequence and the ATC found in wild-type *lacZ* (Fig. 3B) (17). These particular placements were rare. In the screening that yielded pLC104, *lacZ*-utilizing colonies appeared at a frequency of approximately 3%. Plasmid pLC117 was identified in a separate experiment involving more extensive exonucleolytic digestion. In this case, *lacZ*-utilizing colonies were found at a frequency of only approximately 0.01%.⁴

Proteins Produced by pLC104R and pLC117R. The experiment of Fig. 4 uses the "maxcell" technique to display those proteins encoded by pLC104R and pLC117R. Suitably treated maxcells differentially incorporate radioactive amino acids into plasmid-encoded proteins that are easily visualized by autoradiography after polyacrylamide gel electrophoresis (11, 18). pLC104R and pLC117R each direct the synthesis of one protein in addition to β -lactamase. In the case of pLC117R, a protein was produced with a molecular weight of approximately 20,000, consistent with the predicted (from the DNA sequence) size of unglycosylated mature F-IF (8). In the case of pLC104R, the protein produced had a molecular weight of about 23,000, which corresponds to the predicted molecular weight of unglycosylated pre-F-IF.

Fig. 4 also shows the fate of pulse-labeled pLC104R and pLC117R proteins in a maxicell experiment. Densitometry tracing of the gel (not shown) reveals that pre-F-IF was com-



FIG. 4. Proteins encoded by plasmids pLC104R (pre-F-1P) and pLC107B (F-1P). Plasmid proteins were labeled by the modified technique (18). Proteins encoded by pLC104R were labeled for 5 min with [³⁵S]methionine (lane 1) and then chased with nonradioactive methionine for 12 min (lane 2) or 50 min (lane 3). Labeling and chasing were terminated by freezing the cells. After spinning for 2 min in an Eppendorf centrifuge, the cell pellets were suspended in Laemmli sample buffer (13), incubated for 5 min at 100°C, then subjected to analysis by polyacrylamide gel electrophoresis as described (13). Similarly, proteins encoded by pLC107B were labeled for 5 min (lane 4) and chased for 12 min (lane 5) or 50 min (lane 6). Lanes 7, 8, and 9 display the same labeling protocol performed on plasmid pCL103, which encodes only β -lactamase. Plasmid pLC302-2R, which directs synthesis of rabbit β -globin 15000:7500 molecules per cell (11), was likewise analyzed as shown in lanes 10, 11, and 12.

pletely degraded in a 30-min chase (lanes 1 and 3), and F-IF was about 50% degraded in that time (lanes 4 and 6). Although processing of β -lactamase from pre- β -lactamase is evident, pre-F-IF is apparently not processed.

We estimated the level of our F-IF and pre-F-IF synthesis in two ways. First, we measured the amount of radioactivity incorporated during a 5-min pulse into F-IF and pre-F-IF in a maximal experiment. We compared these values with a known standard, namely, rabbit β -globin synthesized by the plasmid pLG302-2R (11) (see Fig. 4). This comparison suggests that, were the F-IF molecules stable, the steady-state levels would be 5000–10,000 molecules per cell. Second, we found that growing cells bearing plasmids pLG117 and pLG104 synthesize about 1200–1400 units of β -galactosidase (19). Assuming that the hybrid F-IF (or pre-F-IF) β -galactosidase molecules have the same specific activity as β -galactosidase, this value represents 2000–10,000 molecules per cell (11). Previous experience with β -galactosidase hybrid proteins modified at their amino termini suggests that they are stable during growth (20).

Antiviral Activity of the Human Fibroblast Interferon Polypeptide Synthesized in *E. coli*. Extracts of bacteria bearing plasmid pLC117R inhibited growth of vesicular stomatitis virus on human fibroblast cells in a typical interferon assay (inhibition of cytopathic effect) (2, 21). This activity was abolished by antibody to F-IF but not by antibody to leukocyte interferon (Fig. 5). Moreover, extracts of bacteria carrying pLC104R, pLC115R, and pBR322 failed to manifest antiviral activity. The antiviral activity directed by pLC117R survives a sojourn at low pH (pH 2.0) or treatment with DNase and RNase, but it is abolished by trypsin treatment (data not shown). Assuming that unglycosylated F-IF is as active as glycosylated F-IF (2×10^4 units/mg (22)), the activity we typically recovered would correspond to approximately 50 interferon molecules per bacterial cell.

DISCUSSION

Our results strongly suggest that our application of the method of Cuarente *et al.* (11) to the F-IF gene isolated by Taniguchi *et al.* (6, 7) has produced two plasmids (pLC1048 and pLC1178) that direct the synthesis of proteins whose primary sequences correspond, respectively, to those of pre-F-IF and F-IF.

† This screening also yielded a third fusion, pLC115. In this case, the portable promoter is abutted to nucleotide 18 of the sequence encoding the F-IF leader some 30 bases from the nearest possible initiator triplet (8). Experiments similar to the experiment of Fig. 4 suggest that, in this case, protein synthesis initiates at the internal ATC located at position 175 in the DNA sequence published by Taniguchi *et al.* (6, 7). This ATC is fortuitously preceded by an SD-like sequence in the F-IF gene. We do not understand how the portable promoter placement in pLC115 enhances the efficiency of utilization of this ATC.

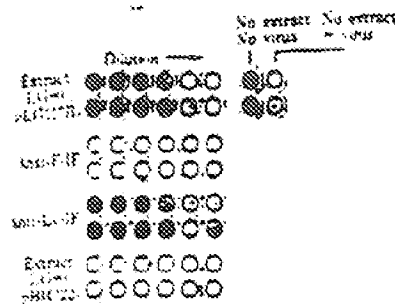


FIG. 5. Characterization of antiviral action of bacterially produced F-IF as assayed *in vitro*. Extracts of bacteria bearing pLG117R were added in 1:2 dilutions to human fibroblast cells (FS-7) growing in microtiter dishes. In two cases, these extracts were pretreated separately with antibody to F-IF and with antibody to leukocyte interferon (Le-IF). The treated cells were challenged with vesicular stomatitis virus and stained with crystal violet (2). Wells containing cells uninfected with virus or protected against viral infection stain darkly with this dye. Also shown are the effects of no extract and an extract of a strain bearing plasmid pBR322.

These plasmids bear a *lac* portable promoter abutted to the ATG encoding the amino-terminal methionine of pre-F-IF (pLG104R) and to the ATG encoding the amino-terminal methionine of F-IF (pLG117R). In each case, these promoter placements were originally recognized by their abilities to efficiently direct synthesis of a F-IF- β -galactosidase hybrid protein. In each case, the distance separating the 5D sequence of the *lac* promoter from the ATG is precisely that found in the case of wild-type *lacZ*. When compared with pBR322, pLG117R and pLG104R each direct synthesis of one new protein of molecular weight approximately 20,000 and 23,000, respectively. These are the sizes expected for unglycosylated proteins with the primary sequences of F-IF and pre-F-IF as predicted from the DNA sequence of Taniguchi et al. (8). In previous cases, we have found that formation of such hybrid ribosome-binding sites, not dissimilar to the ones shown here, have directed correct initiation of protein synthesis (rabbit β -globin, nanian virus 40 tumor (t) antigen, λ repressor) as determined by direct amino acid sequencing (8-11). In all of these cases, the amino-terminal methionine was maintained. We have not determined the amino acid sequence of our bacterially produced F-IF.

Plasmid pLG117R, but no other plasmid described here, directs antiviral activity characteristic of F-IF under our assay condition. The amount of this activity is much lower (only about 1%) than that predicted on the basis of the rate at which the protein is synthesized in our bacteria. We imagine the following possible explanations for this difference.

(i) The protein is rapidly degraded. The pulse-chase experiment of Fig. 5 indicates that the bacterially produced F-IF protein is somewhat unstable under the particular conditions of that experiment. But this degree of instability would not account for the difference between the expected and the observed result. The conditions under which we visualized the proteins (i.e., in microtiter wells) may not accurately reflect the extent of degradation in growing cells.

(ii) Bacterially synthesized F-IF, which is unglycosylated, may have low specific activity in our *in vitro* assay.

(iii) Our method of extraction may not efficiently recover active F-IF. We have not systematically varied growth conditions or methods of extraction.

We have only hints as to why pre-F-IF, synthesis of which is directed by pLG104R, is totally inactive in our assay. It is possible that the unprocessed form is inherently inactive. The microtiter experiments show no indication that pre-F-IF is correctly processed and suggest that it is hyperlabile compared to F-IF. Oxender et al. (23) have described a case (the leucine-specific binding protein) in which the mature form is less sensitive to proteolytic degradation than is the precursor bearing a hydrophobic leader. It is possible that pre-F-IF is exported to the periplasm with or without concomitant cleavage of its leader and is rapidly destroyed there.

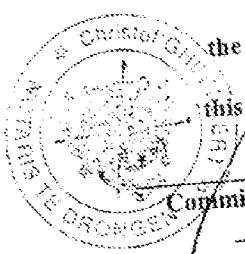
We are indebted to Dr. J. Vilcek for his help and supply of antibodies. The work of T.T. has been supported by a grant from the Cancer Institute, and T.T. thanks Drs. H. Sugano and M. Muramatsu for their encouragement. L.C. is a Postdoctoral Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

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Expression of human fibroblast interferon gene in *Escherichia coli*

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The human fibroblast interferon gene was inserted in a thermoinducible expression plasmid under control of the ϕ lambda P_L promoter. The primary translation products predicted on the basis of the plasmid constructions were hybrid proteins starting with β -lactamase or phage MS2 polymerase information followed by the total preinterferon. On induction, antiviral activity, whose physico-chemical, immunological and biological characteristics closely corresponded to those of authentic human fibroblast interferon, was synthesized. Processing to a size compatible with mature but unglycosylated authentic product was observed.

ON exposure to viruses or other specific inducers most vertebrate cells secrete protein(s) with broad antiviral action known as interferons. Human interferons are being intensively studied for their antiviral¹, anticellular² and immunomodulating³ activities. Clinical trials have been carried out mainly with leukocyte interferon but also with fibroblast interferon, and some promising results have been obtained with both types in the treatment of viral diseases and cancer^{4,5}. Tests with fibroblast interferon have been severely restricted by its very limited availability.

In a previous report¹⁰, we described the construction and characterization of chimaeric plasmids containing human fibroblast interferon (HF-IF) cDNA. Two other groups have constructed plasmids containing either human leukocyte¹¹ or human fibroblast¹² interferon cDNA and in the former case, interferon-related polypeptides, as judged by biological and immunological criteria, were detected in *E. coli* strains harbouring the chimaeric plasmids. We have now inserted the HF-IF coding sequence derived from our original clones into appropriate sites on specifically constructed expression vehicles which contain the strong leftward promoter (P_L) of bacteriophage λ . The functioning of the promoter could be controlled by using host strains which synthesize a temperature-sensitive repressor (cl-ts). We describe here how plasmids containing P_L in front of the HF-IF coding sequence direct the synthesis of polypeptides with human fibroblast interferon activity in *E. coli*.

Construction of plasmids allowing expression of HF-IF

Construction of the different plasmids containing HF-IF DNA under the control of lambda P_L is schematically represented in Fig. 1. The formation and use of acceptor plasmids pPLa2311, pPLa8 and pPLc24 will be published in detail elsewhere (E. Remaut *et al.*, in preparation).

None of the chimaeric plasmids previously described contains an uninterrupted HF-IF gene¹⁰. A complete and continuous coding sequence for HF-IF was reconstituted by inserting an *EcoRI*-*PstI* fragment from pHFIF-6 and a *PstI*-*HaeIII* fragment from pHFIF-7 into the plasmid pPLa2311. From the resulting plasmid, designated pPLa-HFIF-67-1, a *BglIII* fragment was

excised and ligated into the *BamHI* site of the β -lactamase region of pPLa8 in the sense orientation with respect to the promoter. The known nucleotide sequence around the *BamI* *BglIII* junction in this plasmid, pPLa-HFIF-67-12, predicts a polypeptide initiated at the AUG of the β -lactamase part, terminate on a double amber stop codon in the 5'-untranslated region of the HF-IF gene, 23 nucleotides before the HF-initiating AUG (data not shown).

pPLa-HFIF-67-12A19 was derived from pPLa-HFIF-67-1 by deleting a *HindIII* fragment starting within the β -lactamase gene and extending up to three nucleotides before the HF-initiating AUG (Fig. 1). From the known nucleotide sequence the β -lactamase gene (as determined on the progeni pBR322)¹³ and of the HF-IF gene¹⁰, a continuous reading frame starting at the initiating AUG of the β -lactamase gene, running up to the terminating UGA of the HF-IF gene, was predicted. The expected fusion polypeptide consists of 82 amino acid residues of the β -lactamase protein, one amino acid codon at the fused *HindIII* site, and the complete polypeptide (including the putative signal sequence) specified by the HF-gene. The predicted sequence around the junction is: lactamase gene moiety-GUUAAC AUG-HFIF gene, where the GUA triplet codes for amino acid 82 of the β -lactamase protein.

Alternatively, a hybrid plasmid with the controllable lambda P_L promoter in the clockwise orientation was constructed. The acceptor plasmid was pPLc24, which contains the P_L promoter followed by an *EcoRI*-*BamHI* fragment (derived from pMS2 [ref. 14]) containing the ribosome binding site and part of the MS2 polymerase gene. The pPLa-HFIF-67-1 *BglIII* fragment containing the HF-IF gene was inserted into the *BamHI* site of pPLc24, resulting in loss of *BamHI* and *BglIII* sensitivity to formation of *Sau3AI* sites at the joints (Fig. 1). In this plasmid, pPLc-HFIF-67-8, a continuous reading frame starts at the initiating AUG of the MS2 polymerase gene and terminates at the UGA of the HF-IF gene, can be predicted on the basis of the known nucleotide sequences of the MS2 polymerase gene¹⁵ and of pHFIF-6 and pHFIF-7 (ref. 10). The expected fusion protein consists of the N-terminal 98 amino acids of the MS2 polymerase moiety, 27 amino acids coded for by sequences between the *BglIII* site and the initiating AUG of the HF-IF gene, followed by the complete HF-IF coding region.

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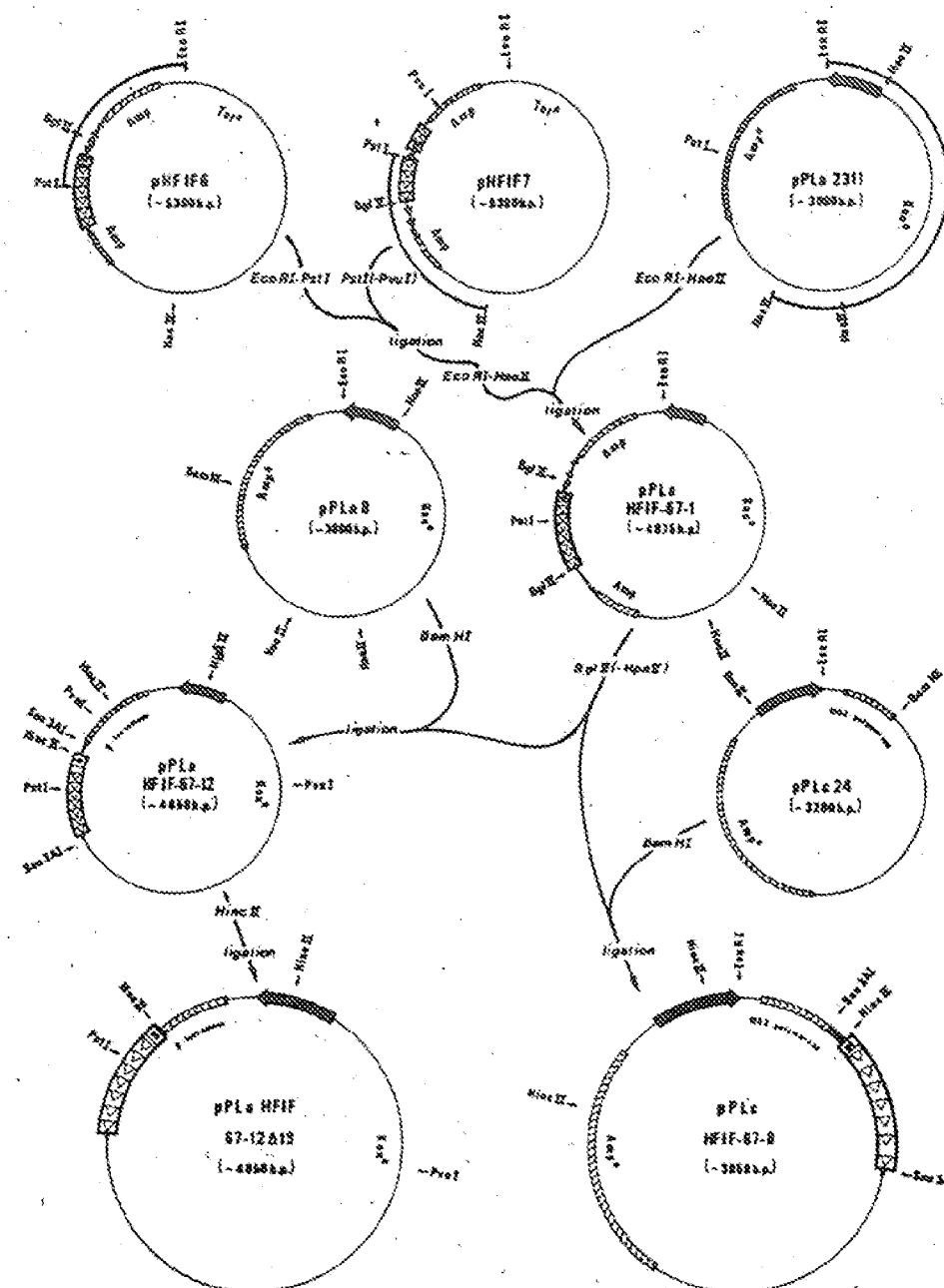


Fig. 1 Schematic outline of the construction of the different plasmids. The HF-IF DNA is indicated by a heavy line, the boxed area being the coding region with the putative signal peptide (S); the triangles indicate the 5'→3' direction of the insert. The heavy arrow indicates the lambda P₁-promoter control element. The ampicillin-resistance region is shown as a shaded area. An interruption of the base line in the insert shows the cross-over region of the inversions of our original HF-IF cDNA-containing plasmids¹⁰. dA-dT tails are indicated by a wavy line. Amp, β-lactamase region coding for resistance to ampicillin and carbenicillin; Kan, region coding for kanamycin resistance. To construct pPLa-HFIF-67-1, pHFIF-6 DNA was cleaved with *Eco*RI and *Pst*I and ligated to pHFIF-7 which had been cleaved with *Pst*I and *Pvu*II. Following ligation, the mixture was digested with *Eco*RI and *Hae*III and ligated to pPLa2311 which had been digested with *Eco*RI and partially with *Hae*III. The segments of the outer circles indicate the fragments retained in the pPLa-HFIF-67-1 construction; only those restriction sites relevant to the constructions are indicated. Transformants were obtained in C600r⁺ (λ) with selection for kanamycin resistance and screened for sensitivity to carbenicillin. The structure of a representative plasmid, pPLa-HFIF-67-1, was confirmed by digestions with *Eco*RI, *Pst*I, *Bgl*II and *Hinc*II (= *Hind*II). To construct pPLa-HFIF-67-12, the HF-IF gene was excised from pPLa-HFIF-67-1 with *Bgl*II, followed by fragmentation of the remaining part of the plasmid with *Hpa*II, which does not cut the HF-IF gene. The DNA was ligated to pPLa8, which had been opened with *Bam*HI. After ligation, the mixture was redigested with *Bam*HI to eliminate recircularized pPLa8 acceptor plasmids. Transformants were obtained in C600r⁺ (λ) by selection for kanamycin resistance. The structure of pPLa-HFIF-67-12 was examined by analysis with *Pst*I and *Hinc*II. To construct pPLa-HFIF-67-12Δ19, pPLa-HFIF-67-12 was partially digested with *Hinc*II and ligated at a low DNA concentration (0.01 μg ml⁻¹). The mixture was then digested with *Xba*I, an isochizomer of *Pvu*II producing 3'-protruding ends¹¹, and religated at low DNA concentration, thereby substantially reducing the probability of religation of the original pPLa-HFIF-67-12 plasmids. The structure of pPLa-HFIF-67-12Δ19 was examined by digestion with *Hinc*II and *Pvu*II. To construct pPLc-HFIF-67-8, the DNA of pPLa-HFIF-67-1 was digested with *Bgl*II and ligated into the *Bam*HI-opened pPLc-24 DNA. The ligation mixture was redigested with *Bam*HI to eliminate religated parental pPLc-24 plasmids. Transformants were selected for resistance to carbenicillin. The insertion and orientation of the *Bgl*II HF-IF fragment was established by *Hinc*II digestion. Restriction enzymes (BioLabs, Boehringer or BRL) were used according to the manufacturer's specifications. Ligations were carried out at 22 °C with T4 DNA ligase in Tris-Cl (50 mM, pH 7.6), MgCl₂ (5 mM), β-mercaptoethanol (7 mM), ATP (50 μM), (ref. 27). All plasmids were subsequently transferred into *E. coli* M5219 (K12 M72 lac⁺ trp⁻ Sm^r [AC1857ΔHI bio 2521])¹⁴. Enzymatic reactions were stopped by heating at 65 °C for 10 min.

including the signal peptide. The predicted sequence around the junction is

Trp-Asp-Leu-Gln-Phe-Arg-Arg-Gln-Pro-
MS2 polymerase gene sequence-DGC-GAG-CGU-CAG-UGU-CGC-AGC-CAA-CGU.

Phe-Glu-Ala-Phe-Ala-Leu-Ala-Gln-Gln-Val-Val-Gly-Asp-Thr-Val-Arg-
UUC-GAA-GCC-UGU-GCU-CUG-GCA-CAA-CAG-GUA-GUA-GGC-GAC-ACU-GUU-CGU.

Val-Val-Asn-His-
GCU-CGC-AAC-AUG-HFIF coding region

The first amino acid, tryptophan, corresponds to position 98 of the MS2 polymerase.

All constructed chimaeric plasmids were transformed into *E. coli* C600 λ and, after characterization, transferred into *E. coli* M5219 (ref. 16), allowing the temperature-dependent controlled expression of the lambda P_L (E. Remaut *et al.*, in preparation).

Detection of IF activity in bacterial extracts

Transcription from the P_L promoter on the plasmids can be turned on by shifting the growing culture from 28 to 42 °C. The synthesis of IF-related product(s) was examined by assaying an S100 extract of the bacteria for antiviral activity (Table 1). The cells were lysed either by lysozyme treatment followed by freeze-thawing or by heating in 1% SDS, 1% β -mercaptoethanol, 5M urea. The extracts of temperature-induced *E. coli* M5219 containing pPLa-HFIF-67-12A19 or pPLc-HFIF-67-8 showed a clear antiviral activity, which was reproducibly higher with the latter plasmid. The same non-induced strains as well as induced M5219 containing a reference plasmid (pPLa8) did not show any detectable activity. In M5219 containing pPLa-HFIF-67-12, trace amounts of antiviral activity were occasionally detected after temperature shift (data not shown), presumably due to a rare reinitiation event. The much higher activity obtained after lysis with the SDS, β -mercaptoethanol, urea mixture indicates a possible nonspecific sticking of the antiviral product(s) to bacterial components, for example, cell membranes or nucleic acids. In parallel experiments in which authentic HF-IF was added to a control bacterial extract obtained by lysozyme treatment, only 10–40% of the activity was recovered.

Low but significant amounts of antiviral activity were detected in the supernatant after osmotic shock of induced M5219 transformed with pPLc-HFIF-67-8 (Table 1). When a more severe method of periplasmic extraction was used (that is, spheroplast formation), some activity was also detected with induced M5219 transformed by pPLa-HFIF-67-12A19. These results suggest that at least some of the bacterial HF-IF may be secreted into the periplasmic compartment, perhaps concomitantly with the

removal of the signal peptide; other explanations, however, cannot be excluded.

Characterization of the bacterial IF activity

The antiviral activity detected in the above-mentioned extracts of induced bacteria was tested for several biological and physical properties characteristic of HF-IF (Table 2). First, the antiviral activity was non-dialysable; after dialysis for 16 h at neutral pH the antiviral activity was retained, albeit often at reduced levels (which was also the case for authentic HF-IF preparations). The observed decrease is presumably due to nonspecific sticking to the dialysis membranes, as HF-IF is known to be rather hydrophobic¹³, and the unglycosylated bacterial form may be even more so. The antiviral activity could be recovered after precipitation with 67% saturated ammonium sulphate, a concentration known to precipitate HF-IF¹³.

When tested for stability at pH 2, a common property of fibroblast and leukocyte interferon¹, bacterial HF-IF proved to remain active (Table 2), although again there was often partial loss of activity, but this was also the case with reconstituted authentic HF-IF controls.

The sensitivity of the bacterial HF-IF activity to protease was tested by treating the diluted bacterial extracts with increasing amounts of trypsin. The activity was abolished at the same concentration of trypsin that abolished the activity of authentic HF-IF added to an inactive control lysate.

HF-IF, in contrast to leukocyte interferon, is stable after heating in 1% SDS, 1% β -mercaptoethanol, 5M urea¹⁹, although we only obtained 10–20% recovery of activity with authentic HF-IF, either alone or in the presence of an inactive bacterial extract (data not shown). The bacterial HF-IF activity remained active in these conditions, as lysis of induced bacteria in this solution resulted in extracts with the highest antiviral titre (Table 1).

The antigenic properties of the *E. coli* IF activity were compared with those of authentic HF-IF. Serial dilutions of goat anti-HF-IF antiserum were incubated with diluted extracts containing bacterial HF-IF activity and with control HF-IF preparations in the presence or absence of an inactive bacterial lysate. The bacterial IF activity was neutralized by the specific antiserum, but some differences were noted in the neutralizing antibody titres for bacterial IF and authentic HF-IF (Table 2). Small differences in neutralization titre were also reported for bacterial leukocyte IF when compared with authentic leukocyte IF¹⁴. This can be explained by a difference either in antigenicity or in specific IF activity of these bacterial proteins relative to authentic IF.

Table 1 Interferon activity in extracts of *E. coli* M5219 transformed by expression plasmids containing the HF-IF coding sequence

Plasmid	Temperature	S100 extracts after lysis by lysozyme and freeze-thawing (I)	Interferon activity (units per ml extract) in S100 extracts after lysis with SDS, β -mercaptoethanol, urea (II)	Periplasmic fraction: osmotic shock (III)	Periplasmic fraction: spheroplast formation (IV)
pPLa-HFIF-67-12A19	28 °C	<3; <2	<30; <100	<2	2
	42 °C	200; 20	200; 2000	<2	10
pPLc-HFIF-67-8	28 °C	<3; <2	<100; <100	<2	<2
	42 °C	200; 50	2,000; 3,000	30	30
pPLa8	42 °C	<3; <2	<100; <100	<2	<2

LB medium (150 ml) was inoculated with 1/500 volume of a fresh seed culture, saturated at 28 °C, and maintained with vigorous shaking at 28 °C until a cell concentration of 2×10^8 ml⁻¹ was reached. Induction was by shifting the temperature to 42 °C and incubation of the cultures for 3 h to a final concentration of $4-6 \times 10^8$ cells ml⁻¹. The cells were collected and washed with Tris-HCl (50 mM, pH 7.4), NaCl (30 mM) and resuspended. Several different extraction procedures were used: I, the bacterial pellet was resuspended in a final volume (4 ml) with HEPES-NaOH (50 mM, pH 7.0), NaCl (30 mM) 3% calf serum, β -mercaptoethanol (5 mM), to which lysozyme (Sigma) was added to 1 mg ml⁻¹. After incubation at 0 °C for 30 min, the suspension was subjected to one or two freeze-thawing cycles. The S100 fraction was prepared by ultracentrifugation at 50,000 r.p.m. for 1 h in a Beckman SW60 Ti rotor. II, The cells were resuspended as in I and lysed in HEPES-NaOH (50 mM, pH 7.0), NaCl (30 mM), 3% calf serum, 1% SDS, 1% β -mercaptoethanol, urea (5 M) at 90 °C for 1–2 min. Clearing by ultracentrifugation was as in I. III, Osmotic shock procedure²¹: the bacterial cell pellet was resuspended in 20% sucrose, EDTA (100 mM), Tris-HCl (100 mM, pH 7.4), to a cell concentration of 1×10^{10} ml⁻¹. After 10 min at 0 °C, the suspension was centrifuged for 10 min at 10,000 r.p.m. The pellet was resuspended in water to a cell concentration of 1×10^{10} ml⁻¹. After 10 min on ice, the suspension was again cleared for 10 min at 10,000 r.p.m. To this osmotic shock supernatant was added HEPES-NaOH (50 mM, pH 7.0), NaCl (30 mM), β -mercaptoethanol (5 mM) and 3% calf serum. IV, Cells were resuspended in 3.6 ml of 0.1 M Tris-HCl, pH 8.0, 20% sucrose, to which 0.4 ml of lysozyme (5 mg ml⁻¹ in EDTA (20 mM)) was added. After incubation for 30 min at 0 °C, the suspension was centrifuged for 10 min at 10,000 r.p.m. The supernatant was adjusted to 3% calf serum. IF activity was measured by a cytopathic effect (CPE)-inhibition assay on human fibroblasts trisomic for chromosome 21 in microtitre trays. The cells were challenged with vesicular stomatitis virus (Indiana strain) and the CPE was recorded at 24 h. All assays included an internal HF-IF reference which was calibrated against the NIH HF-IF reference C2023-902-527. The limit of detection, normally 1 unit ml⁻¹, was often elevated due to toxicity of certain samples (for example, for the samples obtained with method II, the limit of detection was 30–100 units ml⁻¹). The titres are expressed in units ml⁻¹, although it should be noted that they were obtained by 0.5 log₁₀ dilutions.

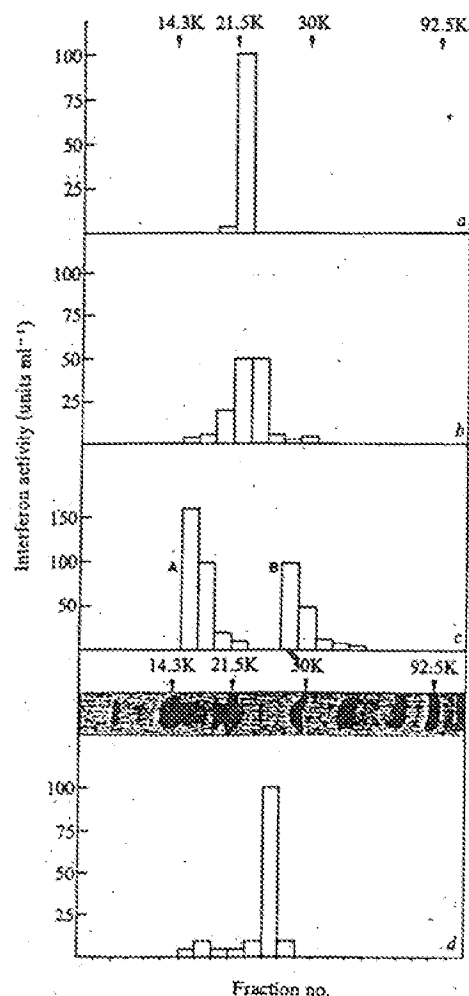


Fig. 2 Polyacrylamide gel patterns of bacterial HF-IF and authentic HF-IF. The following 100- μ l samples were loaded on slots of 2 cm width: a, authentic HF-IF in Eagle's minimal essential medium, 10% calf serum; b, authentic HF-IF in control bacterial extract of MS219/pPLA8 (42°C); c, bacterial extract of MS219/pPLC-HFIF-67-8 (42°C); d, bacterial extract of MS219/pPLA-HFIF-67-12 Δ 19 (42°C). 14 C-labelled protein markers electrophoresed in an equivalent amount of bacterial extract of MS219/pPLC-HFIF-67-8 (42°C) are shown as an insertion in d. Bacterial extracts were prepared by treatment with SDS, β -mercaptoethanol, and urea (compare with procedure II in legend to Table 1). All samples were boiled for 1 min before electrophoresis, which was run according to Laemmli²⁸ in 12.5% polyacrylamide gel. IF activity profiles (a, b, c, d) were obtained after elution of successive 0.5 cm gel slices for 15 h in 500 μ l of 0.5% bovine serum albumin in Tris (0.0125 M), glycine (0.096 M), 0.05% SDS followed by the antiviral IF assay (compare with legend to Table 1). Arrows with molecular weight corresponding to 14 C-labelled markers are indicated on top.

HF-IF is largely species specific, exhibiting little (if any) antiviral effect on heterologous cells¹. Consistent with this property, bacterial IF showed no detectable antiviral protection on cells of monkey, feline, rabbit or mouse origin (Table 3). With respect to the feline cells, bacterial and authentic HF-IF behave differently from human leukocyte interferon.

Further evidence substantiating the presence of active HF-IF in induced *E. coli* extracts was provided by demonstrating the induction of 2', 5'-oligoadenylate (2-5A) synthetase. Kerr *et al.*²⁹ first reported that interferon increases the level of this enzyme in susceptible cells. As shown in Table 4, appropriate bacterial extracts were able to enhance the incorporation of [α - 32 P]ATP in ppp5'A2'p5'A2'p5'A. The 2-5A synthetase-inducing activity of the bacterial extracts was proportional to

their antiviral activity. This bacterial IF activity was likewise neutralized by anti-HF-IF antiserum.

Size estimations of bacterial HF-IF

To estimate the molecular weight of the bacterial IF activity in comparison with authentic HF-IF, bacterial extracts were fractionated by polyacrylamide gel electrophoresis in denaturing conditions and the antiviral activity determined for eluates from successive gel slices (Fig. 2). Whilst authentic HF-IF showed a single peak of activity after electrophoresis, the bacterial IF activity appeared in two different peaks. Very accurate molecular weights could not be assigned because of insufficient resolution of the gel; this was mainly due to an overloading effect

Table 1 Characterization of the bacterial HF-IF activity

	Interferon titre (units ml ⁻¹)
a. Dialysis at neutral pH:	Before After
MS219/pPLC-HFIF-67-8 (42°C) (I)	200 200
(II)	1,000 200
(III)	30 20
MS219/pPLA-HFIF-67-12 Δ 19 (42°C) (I)	200 20
Control HF-IF in MS219/pPLA8 (42°C) (I)	200 100
(II)	200 10
b. Precipitation with (NH ₄) ₂ SO ₄ at 67% saturation:	
MS219/pPLC-HFIF-67-8 (42°C) (I)	100 100
(II)	100 200
MS219/pPLA-HFIF-67-12 Δ 19 (42°C) (I)	100 100
Control HF-IF in MS219/pPLA8 (42°C) (I)	20 20
(II)	30 20
c. pH 2 treatment:	
MS219/pPLC-HFIF-67-8 (42°C) (I)	100 20
(II)	5 5
MS219/pPLA-HFIF-67-12 Δ 19 (42°C) (I)	100 10
Control HF-IF in MS219/pPLA8 (42°C) (I)	1,000 100
d. Heat treatment in 1% SDS, 1% β -mercaptoethanol, 5 M urea (see Table 1)	
	Inactivating and point concentration (mg ml ⁻¹)
e. Trypsin digestion:	
MS219/pPLA-HFIF-67-12 Δ 19 (42°C) (II)	0.03
MS219/pPLC-HFIF-67-8 (42°C) (II)	0.03
(1,000 units ml ⁻¹)	
Control HF-IF in MS219/pPLA8 (42°C) (II)	0.03
(1,000 units ml ⁻¹)	
MS219/pPLC-HFIF-67-8 (42°C) (III)	0.03
(30 units ml ⁻¹)	
Control HF-IF in MS219/pPLA8 (42°C) (III)	0.03
(30 units ml ⁻¹)	
f. Neutralization by antiserum	Neutralization titre (units ml ⁻¹)
pPLC-HFIF-67-8 (42°C) (I)	10 ^{5.5}
(II)	10 ^{5.5}
(III)	10 ^{5.5}
pPLA-HFIF-67-12 Δ 19 (42°C) (I)	10 ^{5.5}
Control HF-IF in extract of MS219/pPLA8 (42°C)	10 ^{5.5}
pPLC-HFIF-67-8 (42°C) (II): elution peak A	10 ^{4.5} ; 10 ^{4.5}
elution peak B	10 ^{4.5} ; 10 ^{4.5}
Control HF-IF eluted from polyacrylamide gel	10 ^{5.5}
Control HF-IF	10 ^{5.5}
Control leukocyte IF	<10
g. Anti-viral activity in heterologous cells (see Table 3)	
h. 2-5A synthetase induction (see Table 4)	

The following experimental methods were used for the characterization: a, dialysis at neutral pH took place overnight at 4°C against phosphate buffered saline (PBS). b, Two volumes of a saturated (NH₄)₂SO₄ solution were added to one volume of extract. After 30 min on ice, the pellet was centrifuged at 12,000g for 10 min and redissolved in PBS. c, The bacterial extracts were either dialysed for 15 h against glycine-HCl (50 ml, pH 2.2), followed by dialysis against PBS for 3 h, or acidified with HCl, followed by neutralization with NaOH. After removal of the precipitate the antiviral activity was determined. e, Trypsin digestion was for 1 h at 37°C with serial dilutions of the enzyme added to the diluted extract. The lowest trypsin concentration that completely abolished the antiviral activity is indicated. f, The antibody neutralization assays were carried out essentially as described by Havell *et al.*³⁰. About 10 IF units ml⁻¹ of the preparations were incubated for 1 h at 37°C with serial dilutions of goat anti-HF-IF antiserum, after which the residual antiviral activity was determined. Values are presented as neutralizing titres, that is, the highest dilution of antiserum which neutralized the protective effect of IF by 50% multiplied by the interferon titre of the sample assayed. Roman numerals in parentheses refer to the extraction methods described in Table 1.

Table 3 Antiviral protection of bacterial IF activity and authentic interferons

	Interferon activity (units per ml), assayed on					
	Human T-21	Human VGS	Monkey BSC-1	Rabbit primary kidney	Feline lung	Mouse L-929
M5219/pPLc-HFIF-67-8 (42 °C) (III)	3,000	300	<100	<100	<100	ND
(III)	30	<10	<10	<10	<10	ND
M5219/pPLc-HFIF-67-8 (42 °C) (III) elution peak A	2,000	200	<10	<10	<10	<10
M5219/pPLc-HFIF-67-8 (42 °C) (III) elution peak B	2,000	500	<10	<10	<10	<10
Human fibroblast interferon	3,000	300	30	30	<3	<2
Human leukocyte interferon	5,000	500	30	10	1,000	30
Human immune (type II) interferon	3,000	1,000	10	<3	<3	<2
Mouse L-929 interferon	<2	<2	<2	<2	<2	500

The antiviral activity was assayed as described in the legend to Table 1, except that the titres were directly determined from the dilution end points. T-21 are human fibroblasts trisomic for chromosome 21; VGS are normal human diploid fibroblasts²³. Feline lung cells were obtained from Flow Laboratories (cat. no. 0-10907). ND, not determined.

which resulted in a different migration of the proteins, as revealed by internal ¹⁴C-labelled protein markers. Both peaks were neutralized to the same extent with anti-HF-IF antiserum (Table 2) and did not show detectable IF activity on heterologous cells (Table 3). The first peak, corresponding to an approximate molecular weight (MW) of 15,000–18,000, may have arisen by haphazard proteolytic cleavage of the fusion protein, or by limited *bona fide* processing at the now internal signal peptide, or by a combination of both processes. As shown previously, the absence of the carbohydrate moieties results in a protein which migrates in polyacrylamide gel to a position of about 4,000 MW below the authentic glycosylated HF-IF¹⁰. The 15,000–18,000-MW component, clearly present in *E. coli* M5219/pPLc-HFIF-67-8 extract, could also be detected at low but still significant levels in M5219/pPLc-HFIF-67-12A19 extracts. The second peak, with an apparent higher molecular weight, could correspond to the fused prokaryotic HF-IF poly-

peptide, or a slightly processed form. The tentative identification of the slower moving HF-IF activity peak as the fusion protein is strengthened by a different migration of the activity in the extract of M5219/pPLc-HFIF-67-12A19 (with a predicted fusion protein of about 28,000 MW) compared to the M5219/pPLc-HFIF-67-8 extract (with a predicted fusion protein of about 33,000 MW) (Fig. 2). The fusion proteins may themselves have some activity or be processed to an active product at the time when the bacterial extract (or gel eluate) is applied onto the human cells for the antiviral assay.

Conclusion

We have demonstrated the expression of HF-IF activity in *E. coli*. This synthesis depends on the presence of the HF-IF cDNA gene in the appropriate orientation and of the controlled induction of transcription from the P_L promoter. The antiviral activity obtained from *E. coli* is due to the presence of polypeptide(s) which for all physicochemical, biological and immunological characteristics tested closely resembles authentic HF-IF. Polyacrylamide gel electrophoresis resolved the bacterial IF activity into two different size classes; the smaller component presumably resulted from a post-translational cleavage of the fusion proteins.

The HF-IF produced in *E. coli* is still low in titre (about 100 units per 5 × 10⁸ cells ml⁻¹), but undoubtedly this can be improved by better plasmid constructions. Thus, we hope to produce sufficient quantities of bacterial HF-IF to compare its biological and pharmacological properties with those of authentic glycosylated HF-IF and of bacterial leukocyte IF, and perhaps to evaluate its potential clinical applications.

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Table 4 2-5A synthetase inducing activity of bacterial lysates and its neutralization by anti-HF-IF antiserum

	³² P incorporation (in c.p.m.) into the 2-5A trimer (background subtracted)
a. M5219/pPLc-HFIF-67-8 (42 °C) (III) (30 units ml ⁻¹)	3,618
Control HF-IF added to M5219/pPLc (42 °C) (III) (30 units ml ⁻¹)	3,693
M5219/pPLc (42 °C) (III) (<2 units ml ⁻¹)	(~1,368)
Control HF-IF (18 units ml ⁻¹)	1,120
(60 units ml ⁻¹)	4,338
(180 units ml ⁻¹)	10,273
(600 units ml ⁻¹)	21,698
b. Control HF-IF (100 units ml ⁻¹)	12,468
M5219/pPLc (42 °C) (I)	1,011
M5219/pPLc-HFIF-67-12A19 (42 °C) (I)	42,193
plus 1 n.u. per ml anti-HF-IF	29,260
plus 100 n.u. per ml anti-HF-IF	727
M5219/pPLc-HFIF-67-8 (42 °C) (I)	17,478
plus 1 n.u. per ml anti-HF-IF	12,515
plus 100 n.u. per ml anti-HF-IF	7,992
plus 10000 n.u. per ml anti-HF-IF	2,517

All samples were diluted sixfold (a) or tenfold (b) before assay. Antiviral titres (before dilutions) are given in parentheses. Neutralization with goat anti-HF-IF antiserum was at 37 °C for 1 h; n.u., neutralizing units. Roman numerals in parentheses refer to the extraction methods described in Table 1 legend. The obtained values are listed after subtraction of the endogenous background activity: 3,242 c.p.m. in a and 2,073 c.p.m. in b. The 2-5A synthetase assay was modified from Kimchi et al.²⁴ and Miska et al.²⁵. Confluent monolayers of HeLa cells in microtitre plates (96 wells) were treated with the diluted bacterial extract or with control HF-IF for 20 h. After cooling and washing with NaCl (140 mM), Tris-HCl (35 mM, pH 7.5), the cultures were lysed in 5 µl (a) or 10 µl (b) of 0.5% NP40, PMSF (1 mM), NaCl (140 mM), Tris-HCl (35 mM, pH 7.5). After shaking vigorously for 20 min at 0 °C, the lysates were collected and centrifuged for 20 min at 18,000g. 3.5 µl of the supernatant was incubated for 2 h at 31 °C in 6 µl of KOAc (100 mM), Mg(OAc)₂ (25 mM), HEPES-KOH (10 mM, pH 7.4), ATP (5 mM), fructose-1,6-bis-phosphate (4 mM), DTT (1 mM), poly I-C (20 µg ml⁻¹) and 2 µCi of ³²PATP (400 Ci mmol⁻¹). The reaction was stopped by heating for 3 min at 95 °C and the samples were treated with 150 units ml⁻¹ of calf intestine alkaline phosphatase (Boehringer) at 37 °C for 1 h. After clearing, 1 µl was spotted on PEI-cellulose thin-layer plates and chromatographed in 1 M acetic acid for 2–3 h. The plates were autoradiographed and the incorporation of ³²P in the 2-5A trimer was determined.

一

Gel electrophoresis - same substrate + controls HF-2F
Patrick - Stevenson

gel I

2x20 mm

1 mm thick

12.5% AA. ammonop gel

5% AA. spacer gel

Lane 1

200 µl in 2.5 mm slot

100 µl control HRP (100% control) in 100% HF-2F

+ 100 µl 1% bovine BSA sample buffer

+ 100 µl 1% BSA sample buffer

+ BSA

Lane 2

200 µl in 2.5 mm slot

100 µl extract 243 // P/6

↳ SDS / 100% HF-2F

100 µl control HRP (100% control) in 100% HF-2F

(100% control) in 100% HF-2F

+ 100 µl 1% bovine BSA sample buffer

+ 100 µl 1% BSA sample buffer

+ BSA

Lane 3

200 µl in 2.5 mm slot

100 µl extract 191 // 4/16

↳ SDS / 100% HF-2F

(SDS - 100% HF-2F) in 100% HF-2F

sample 191

+ BSA

Lane 4

100 µl extract 191 // 4/16 cf. Lane 3

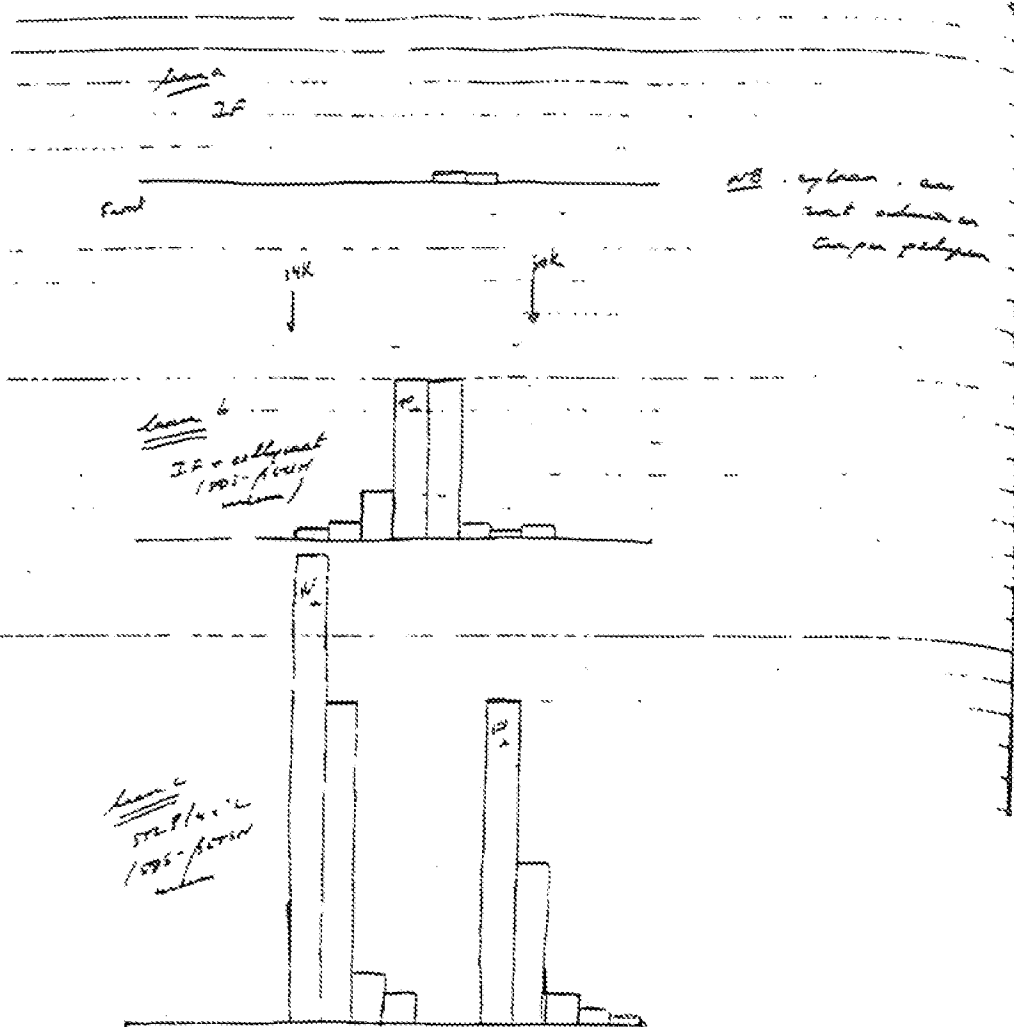
+ 100 µl 1% BSA sample buffer

+ BSA

Lane 5 cf. 4

Lane 6 " machine in bovine BSA sample buffer

all 6 lanes 2.5 mm slots



	W/S	E/S/T	T/L	primary collect material	PRK	Refine	BSC-2	C914
mat C ₁	2.3	2.5	3.3	<1.2	<1.2	<1.2	<1.2	<1.2
mat C ₂	2.7	2.5	3.3	<1.2	<1.2	<1.2	<1.2	<1.2
mat C ₃ 2.1/1.6	2.5	2.5	2.1	<1.2	<1.2	<1.2	<1.2	<1.2
2ZF	2.7	2.5	3.7	1.0	3.0	1.5	1.5	1.5
IF	2.7	2.3	3.5	2.5	0.5-0.7	1.5	0	0
mat IF	3.2	3.3	3.5	<0.5	<0.5	1.0	0	0
from IF	0	0	0	0	0	0	0	1.2

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3	0.05	3	0.05	3	0.15
4	0.05	4	0.05	4	0.15
5	0.05	5	0.05	5	0.15
6	0.05	6	0.05	6	0.2
7	0.05	7	0.05	7	0.2
8	0.05	8	0.2	8	1.2
9	0.05	9	1.2	9	1.0
10	0.05	10	1.2	10	0.05
11	0.05	11	0.05	11	0.05
12	0.05	12	0.05	12	0.05
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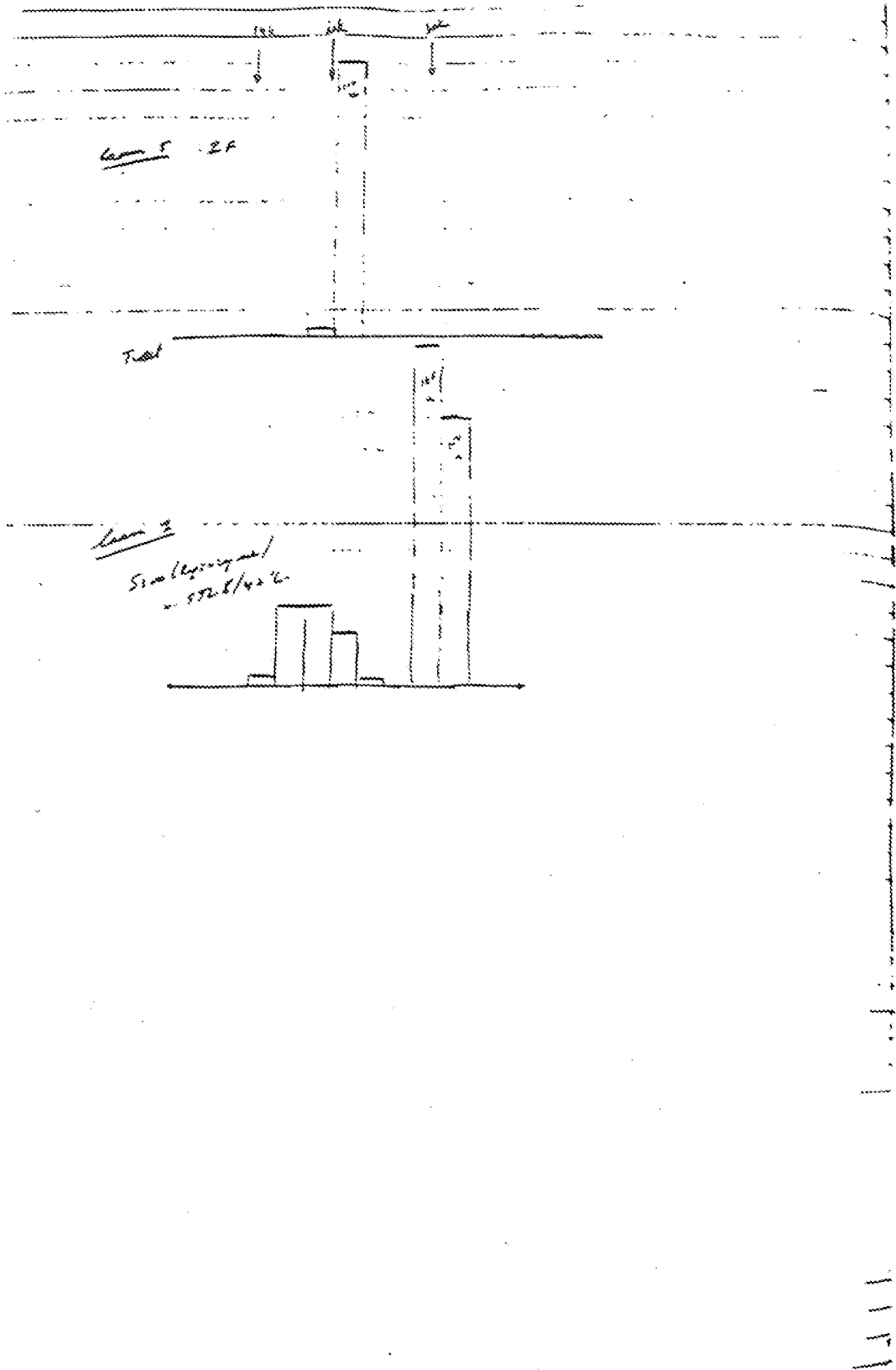
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sample 66 10^{6.7}
sample 612 10^{6.7}
HFIC 10^{6.7}

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38 Leadbury in IP



Simulation of T-2

Loan 5	Loan 6	Loan 7	Loan 8	Loan 9
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2/ 0.5	15/ 0.5	28/ 0.5	46/ 0.5	54/ 0.5
3/ 0.5	16/ 0.5	29/ 0.5	47/ 0.5	55/ 0.5
4/ 0.5	17/ 0.5	30/ 0.5	48/ 0.5	56/ 0.5
5/ 0.5	18/ 0.5	31/ 0.5	49/ 0.5	57/ 0.5
6/ 0.5	19/ 0.5	32/ 0.5	50/ 0.5	58/ 0.5
7/ 0.5	20/ 0.5	33/ 0.5	51/ 0.5	59/ 0.5
8/ 0.5	21/ 0.5	34/ 0.5	52/ 0.5	60/ 0.5
9/ 0.5	22/ 0.5	35/ 0.5	53/ 0.5	61/ 0.5
10/ 0.5	23/ 0.5	36/ 0.5	54/ 0.5	62/ 0.5
11/ 0.5	24/ 0.5	37/ 0.5	55/ 0.5	63/ 0.5
12/ 0.5	25/ 0.5	38/ 0.5	56/ 0.5	64/ 0.5
13/ 0.5	26/ 0.5	39/ 0.5	57/ 0.5	65/ 0.5

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 49 : 2.7

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unveränderliche 45 : 2.7
 49 : 2.7
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~~13-52 separation gel~~
~~52 separation gel~~

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 (rem old) \rightarrow S_{22} (4x152gms) 2218/1752/41°C
 temp 721 = 1.2

+ 3F8

leaven 2 66 gel sample 193/15 + 33 gel 30 handhaffen 2F
 (rem old) \rightarrow S_{22} (4x152gms) 2218/1752/41°C
 temp 721 = 1.2

+ 3F8

leaven 3 66 gel sample 193/15
 (rem old) \rightarrow S_{22} (4x152gms) 2218/1752/41°C
 mix: SDS - β DTX - urea
 temp 721 = 2.35

+ 3F8

leaven 4 5 gel 19°C - marker + 4 gel 35°C - DTHV - calibration
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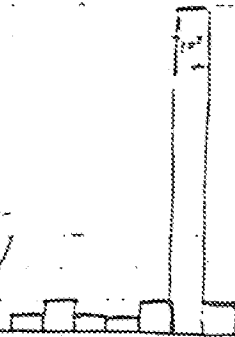
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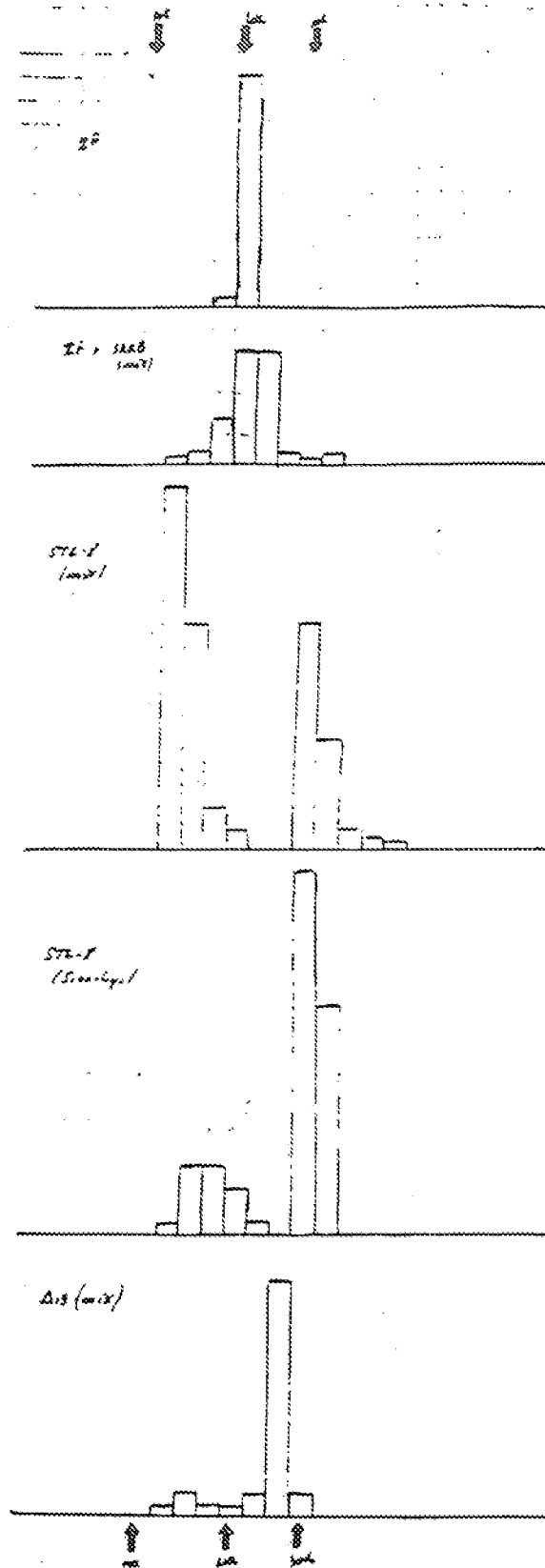
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and horizontal by rest



of 24.

<u>Team 2</u>	<u>Team 3</u>	<u>Team 4</u>	<u>Team 5</u>
20 abnormal ppl 160/2.5	20 abnormal ppl 160/2.5	20 abnormal ppl 160/2.5	20 abnormal ppl 160/2.5
11/0.5	14/0.5	27/0.5	41/0.5
2/0.5	15/0.5	28/0.5	42/0.5
3/0.5	16/0.5	29/0.5	43/0.5
4/0.5	17/0.5	30/0.5	44/0.5
5/0.5	18/0.5	31/0.5	45/0.5
6/0.5	19/0.5	32/0.5	46/0.5
7/0.5	20/0.5	33/0.5	47/0.5
8/0.5	21/0.5	34/0.5	48/0.5
9/0.5	22/0.5	35/0.5	49/0.5
10/0.5	23/0.5	36/0.5	50/0.5
11/0.5	24/0.5	37/0.5	51/0.5
12/0.5	25/0.5	38/0.5	52/0.5
13/0.5	26/0.5	39/0.5	53/0.5
14/0.5	27/0.5	40/0.5	54/0.5
15/0.5	28/0.5	41/0.5	55/0.5
16/0.5	29/0.5	42/0.5	56/0.5
17/0.5	30/0.5	43/0.5	57/0.5
18/0.5	31/0.5	44/0.5	58/0.5
19/0.5	32/0.5	45/0.5	59/0.5
20/0.5	33/0.5	46/0.5	60/0.5

Team 4: not a participant

Team 5: complete independent - ask if full name
as possible
of the sample size